Biomaterials 76 (2016) 196–207

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

siRNA delivered by EGFR-specific scFv sensitizes EGFR-TKI-resistant human lung cancer cells

Yuan Lu [a, 1], Li Liu [a, b, 1], Yuan Wang [a, 1], Fakai Li [a], Jian Zhang [a], Mingxiang Ye [a], Hu Zhao [c], Xiang Zhang [d], Mi Zhang [e], Jing Zhao [d], Bo Yan [d], Angang Yang [f], Huasong Feng [e, ***], Rui Zhang [d, **], Xinling Ren [a, *]

[a] Department of Respiratory Medicine, Xijing Hospital, Fourth Military Medical University, 127 Changle West Road, Xi'an 710032, China
[b] Department of Geriatrics, The Central Hospital of Xianyang, 78 Renming East Road, Xianyang 712000, China
[c] The Organ Transplant Institute, The Fuzhou General Hospital (DongFang Hospital), Xiamen University, 156 West Ring Road, Fuzhou 350025, China
[d] The State Key Laboratory of Cancer Biology, Department of Biochemistry and Molecular Biology, Fourth Military Medical University, 169 Changle West Road, Xi'an 710032, China
[e] Department of Respiratory Medicine, Naval General Hospital, 6 Fucheng Road, Beijing 100048, China
[f] The State Key Laboratory of Cancer Biology, Department of Immunology, Fourth Military Medical University, 169 Changle West Road, Xi'an 710032, China

ARTICLE INFO

Article history:
Received 10 June 2015
Received in revised form
11 October 2015
Accepted 14 October 2015
Available online 23 October 2015

Keywords:
NSCLC
Tyrosine kinase inhibitors (TKIs)
Resistance
scFv
Targeted therapy
RNA interference

ABSTRACT

The overexpression of epidermal growth factor receptor (EGFR) is closely associated with a poor outcome in non-small cell lung cancer (NSCLC), and EGFR is an ideal biomarker for the targeted therapy of NSCLC. Although patients with EGFR-activating mutations respond to EGFR tyrosine kinase inhibitors (EGFR-TKIs), they eventually acquire resistance, which typically results from a secondary EGFR mutation or the activation of other signaling pathways. Novel approaches to overcome or prevent EGFR-TKI resistance are clinically important. In this study, we developed an EGFR-scFv-arginine nonamer peptide fusion protein, s-9R, as an siRNA carrier. Here, we show that s-9R effectively and specifically delivers EGFR-siRNAs, KRAS-siRNA and MET-siRNA into NSCLC cells and silences the expression of target genes. The sensitivity of NSCLC cells to gefitinib was restored after treatment with the s-9R/siRNA complex, and the apoptosis rates of the treated cells were significantly higher than those of the control groups. Furthermore, the co-administration of s-9R/siRNA and gefitinib successfully suppressed the progression of H1975 xenograft tumors and extended the life span of tumor-bearing nude mice. Collectively, the results of this study provide not only a new scFv derivative for delivering siRNA into EGFR-overexpressing, TKI-resistant NSCLC cells but also a novel method for overcoming TKI resistance.

1. Introduction

Epidermal growth factor receptor (EGFR) is overexpressed in approximately 60% of non-small cell lung cancer (NSCLC) cases. By binding to ligands, EGFR regulates downstream signaling pathways, most importantly the rat sarcoma (RAS) and phosphoinositide 3-kinase (PI3K) pathways, and it plays an important role in tumor cell proliferation, survival, migration and metastasis [1]. Tyrosine kinase inhibitors (TKIs), particularly reversible inhibitors such as erlotinib and gefitinib, are widely used in NSCLC patients harboring EGFR-activating mutantions. However, patients who initially responded to EGFR-TKI treatment, eventually developed resistance to reversible EGFR-TKIs, which may be due to secondary EGFR mutations or other resistance mechanisms [2]. The T790M point mutation in exon 20 of EGFR, which increased the affinity for ATP and blocks the binding of TKIs to EGFR, is responsible for 50% of all resistance to reversible TKIs [3]. In addition, Kirsten rat sarcoma viral oncogene (KRAS) gene mutations, which result in the stimulus-independent activation of KRAS and downstream effectors and are proposed to be responsible for the primary resistance to reversible inhibitors, exist in approximately 15–30% of NSCLC tumors [4]. Mesenchymal to epithelial transition factor (MET) gene amplification also leads to EGFR inhibitor resistance by driving the

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: fenghs99@163.com (H. Feng), ruizhang@fmmu.edu.cn (R. Zhang), majrenxl@fmmu.edu.cn (X. Ren).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.biomaterials.2015.10.036

HER3-dependent activation of PI3K, which occurs in approximately 20% of the NSCLC patients who initially responded to reversible inhibitors and approximately 7% of TKI-naïve patients [5]. Other mechanisms, including the epithelial-mesenchymal transformation (EMT), the conversion of NSCLC to SCLC, AXL upregulation and crk-like protein (CRKL) amplification, also play a role in the development of TKIs resistance [6].

New generation inhibitors have been developed to overcome T790M resistance by covalently binding with ATP. Unfortunately, most irreversible inhibitors are associated with severe adverse effects and the emergence of novel resistant mutations in NSCLC cells [7–9]. Other therapeutic strategies to overcome the EGFR-TKI resistance caused by MET amplification or KRAS-activating mutations have also been tested in clinical trials, but most of these strategies involve small-molecule inhibitors or anti-EGFR antibodies, which produce severe off-target effects [10]. Furthermore, the ability to modulate TKI resistance has been complicated by the fact that NSCLC simultaneously exhibits multiple resistance mechanisms.

Silencing gene expression by siRNA might be a potent tool for cancer treatment. However, the lack of efficient and specific delivery methodologies is the main obstacle for the clinical success of siRNA-based cancer therapeutics [11]. Studies have shown that antibody-mediated targeted delivery is an effective method for delivering and internalizing siRNA into particular cells [12–15]. A single chain antibody (scFv) composed of variable regions of VH and VL chains connected in tandem via a short linker is an ideal tool for delivering therapeutic reagents because it lacks both the constant regions and the Fc domains, making it much smaller than the intact antibody; thus, it has stronger penetrating capability and lower immunogenicity [16]. However, successful delivery systems also require efficient vehicles to carry therapeutic agents. Arginine-rich cell-penetrating peptides can bind and transduce nucleic acids, such as DNA and siRNA, both *in vitro and in vivo* through electrostatic interactions. The 9R motif, a typical cell-penetrating peptide, is more efficient for cellular uptake than other oligomers containing fewer arginine residues or other cationic amino acids such as histidine, lysine or ornithine [17].

Given that lung cancer is the most common cancer and the leading cause of cancer mortality worldwide [18], in the present study, we attempted to overcome EGFR-TKI resistance in EGFR-positive NSCLC cells by knocking down the expression of EGFR, MET and KRAS using the powerful scFv-based delivery system described above. We generated a single-chain EGFR antibody (scFv, abbreviated as "s"), and fused it to a nine-mer arginine (9R) peptide (s-9R). Our data show that s-9R efficiently delivers siRNA into EGFR-overexpressing lung cancer cells, and our strategy potentiates the effect of gefitinib on human NSCLC lines bearing TKI-resistant mutations both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Plasmid construction and protein purification

The genes encoding scFv and s-9R were designed based on the amino acid sequences of nimotuzumab. A linker (Gly4Ser3) was connected to the VH and VL chains, and the nine-mer arginine-encoding sequence was included in the 3′scFv sequence to obtain the s-9R fusion gene. To track the scFvs, we also included a 6 × His-encoding sequence in the 3′scFv sequence, which was synthesized by Augct Co. (Beijing, China). For bacterial expression, the scFv and s-9R sequences were isolated as a BamHI/XhoI fragment from the T vector and inserted into a BamHI/XhoI-digested plasmid, pGEX-4T-1 (Novagen, Uppsala, Sweden). Single colonies of *E. coli* BL21 (DE3) carrying the manufactured plasmid pGEX-4T-1-scFv for the expression of the EGFR-specific scFv protein were grown overnight at 37 °C in 2 × YT medium supplemented with 100 μg/ml ampicillin. The cultures were then diluted 100-fold in the same medium and incubated at 37 °C until they reached an OD600 of 0.4–0.6. The cultures were then induced with 0.1 mM isopropyl-1-thio-β-galactopyranoside (IPTG) at 30 °C for 5 h. The cells were harvested via centrifugation (5000 g, 4 °C, 15 min), washed in ice-cold phosphate-buffered saline (PBS), and then resuspended in binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.4). The soluble cell lysates were centrifuged at 12,000 g for 20 min after 30 min of sonication. Following the manufacturer's instructions, the supernatant was then applied to a glutathione S-transferase (GST)-affinity column (GE Healthcare, Uppsala, Sweden). The fusion proteins were eluted from the GST-affinity column using 10 mM reduced glutathione dissolved in 50 mM Tris–HCl (pH 8.0) at room temperature. The purified sample was dialyzed against PBS (pH 8.0) for 24 h at 4 °C and then incubated with 25-fold diluted thrombin (Novagen, Darmstadt, Germany) at room temperature for 8 h. Finally, the solutions were filtered in a GST affinity column to remove the GST tag. The concentrations of the resultant fusion proteins were determined using a bicinchoninic acid assay (Pierce, Rockford, IL, USA), and specific proteins were identified by immuno-blotting with an anti-6 × His mouse monoclonal antibody (Qiagen, Valencia, CA, USA).

Detailed information about other experimental procedures is provided in the Supplementary Materials.

3. Results

3.1. Purification of the EGFR-targeting scFv and s-9R

For our study, we constructed the EGFR-targeting scFv and s-9R based on nimotuzumab by sequentially fusing the VH and VL chains with a G4S linker and a 6 × His.tag. s-9R was also equipped with a nine-mer arginine tail to carry the siRNA (Fig. 1A). The sequences encoding scFv and s-9R were cloned into the pGEX-4T-1prokaryotic expression vector (Fig. 1B). Following incubation in the presence of IPTG, transformed *E. coli* cells expressed the recombinant single-chain antibodies fused to the GST tag. The resulting fusion proteins were cleaved by thrombin at room temperature and purified using GST-affinity chromatography. We detected the fusion proteins using SDS-PAGE and observed that the molecular weight of scFv and s-9R was approximately 26 kDa, which is consistent with the expected size. The purity of the fusion proteins was greater than 90% based on optical density analyses of the bands (Fig. 1C), and the scFvs fused with 6 × His peptide tag were further identified by Western blotting using an anti-6 × His.tag antibody (Fig. 1D).

3.2. The scFv and s-9R fusion proteins retain the EGFR-binding ability and are internalized by EGFR-positive lung cancer cells

The binding ability of the scFv and s-9R to EGFR was confirmed using ELISA. As shown in Fig. 2A, the two fusion proteins retained antibody affinity for recombinant EGFR proteins but not the negative control protein BSA. The rate of antibody-immobilized coupling gradually decreased as the concentrations of scFv and s-9R decreased. For the flow cytometry (FCM) assay, the lung cancer cell lines were incubated with the fusion proteins, and the cellular fluorescence intensity was monitored. The FITC fluorescence of EGFR-positive A549, H1975 and H1993 cells exceeded that of EGFR-negative H69 cells, which showed only background fluorescence, by 15-fold; the proportion of FITC-stained H69 cells did not exceed 5% on average (Fig. 2B). The shift in the cellular FITC peak in the FCM assay represents an increase in the number of fluorescence-positive cells. This shift was not observed in the EGFR-deficient

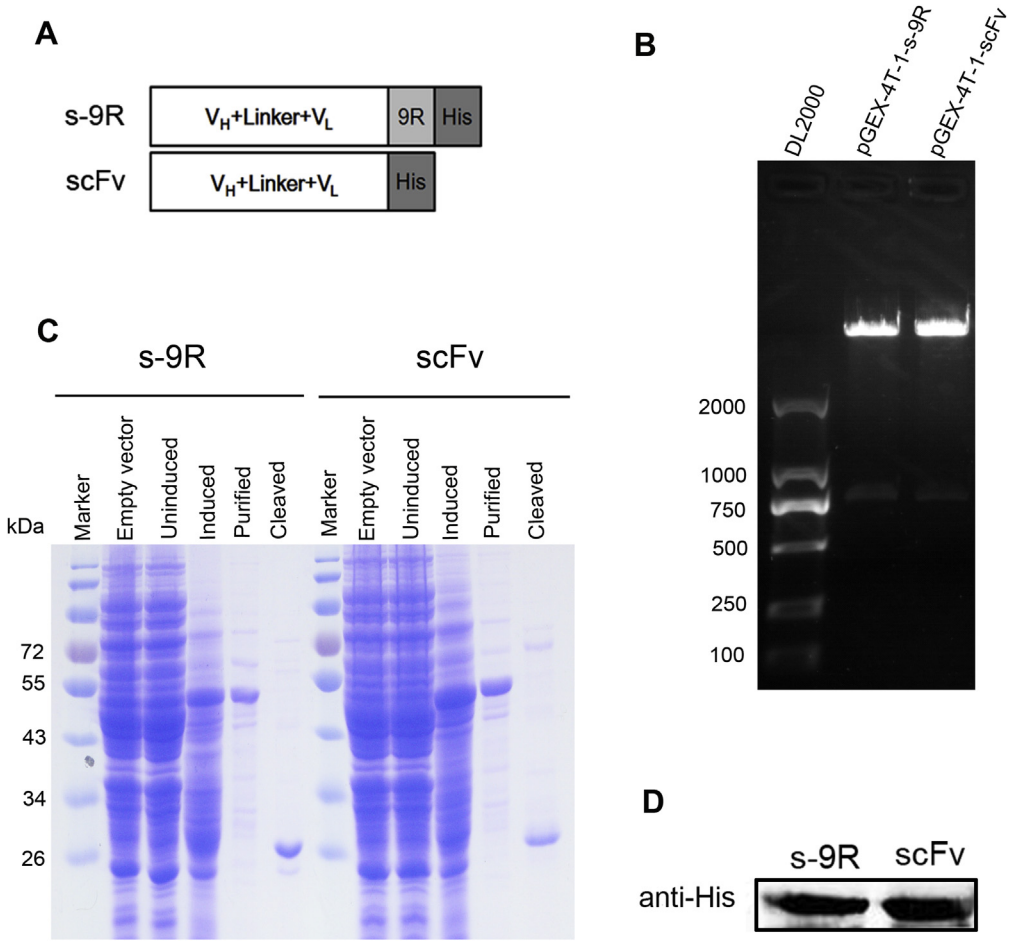

Fig. 1. Expression and purification of s-9R and scFv. (A) Schematic diagram of s-9R and scFv. (B) Restriction enzyme digestion result of the s-9R and scFv expression plasmids in pGEX4T-1. (C) SDS-PAGE of expressed and purified s-9R and scFv fusion proteins. (D) Identification of purified s-9R and scFv fusion proteins by Western blot.

H69 cell groups incubated with either of the single-chain antibodies. However, the peak occurred in EGFR-positive A549, H1975 and H1993 cells incubated with scFv or with s-9R (Fig. 2C). To confirm that the fusion proteins can bind to and be internalized by EGFR-positive cells, we incubated NSCLC cells with scFv or s-9R for 6 h before immunofluorescence staining and used Cy3-conjugated secondary antibody to locate both of single chain antibodies in target cells. The A549, H1975 and H1993 cells incubated with the fusion proteins showed red fluorescence from the Cy3 dye. The signal was observed on the cell membranes, dispersed in the cytoplasm and even in the nuclei of the EGFR-positive cells. Fluorescence signals were not detected in the H69 cells in any of the groups. To rule out the possibility of nonspecific effects, the cells were also transfected with EGFR-siRNA using Lipofectamine^{2000} 48 h before the fusion proteins were added. The pre-transfection almost completely abrogated the cellular uptake of the fusion proteins (Fig. 2D). Therefore, the fusion proteins specifically target EGFR-positive lung cancer cells.

3.3. The s-9R fusion protein can target and deliver siRNA into EGFR-positive lung cancer cells

The amount of the s-9R fusion protein mixed with the nonspecific nucleotide fragments negatively correlated with the migration rate of the nucleotide fragments. In contrast, nucleotide fragments mixed with scFv or BSA showed no significant change in gel mobility (Fig. 3A). To determine the molar ratio between the s-9R and siRNA in additional experiments, a colorimetric binding assay was performed. Specifically, each s-9R molecule was bound to approximately three FAM-labeled siRNAs (FAM-siRNAs) (Fig. S1). To determine whether the s-9R fusion protein could specifically deliver siRNA into EGFR-positive cells, FAM-siRNA was added to A549, H1975, H1993 and H69 cells in combination with scFv, s-9R or BSA before the FCM analysis. The EGFR-positive cells were unable to internalize the FAM-siRNA that was mixed with scFv or BSA. Moreover, the EGFR-positive cells effectively internalized the FAM-siRNA that was mixed with the s-9R fusion protein, but the H69 cells did not (Fig. 3B and C). Lipofectamine^{2000}-mediated transfection served as a positive control for siRNA delivery. We next evaluated the specificity of siRNA delivery with LSCM. FAM-siRNA was premixed with s-9R, scFv or BSA for 30 min and then added to the cell culture medium. The cells were then incubated for 6 h Lipofectamine^{2000}-mediated transfection of FAM-siRNA was used as a positive control. As shown in Fig. 3D, the green fluorescence of FAM-siRNA, which was mixed with s-9R and Lipofectamine^{2000}, was located on the surface of the EGFR-positive lung cancer cells and scattered throughout the entire cytoplasm, whereas pre-incubation with scFv and the irrelative protein BSA did not result in cellular FAM fluorescence. None of the H69 cells fluoresced when treated with s-9R/FAM-siRNA or scFv/FAM-siRNA, although the FAM-siRNA that was transfected with Lipofectamine^{2000} could be detected.

3.4. Specific siRNAs delivered by s-9R inhibited KRAS, EGFR and MET gene expression

In this study, we used three NSCLC cell lines that harbored

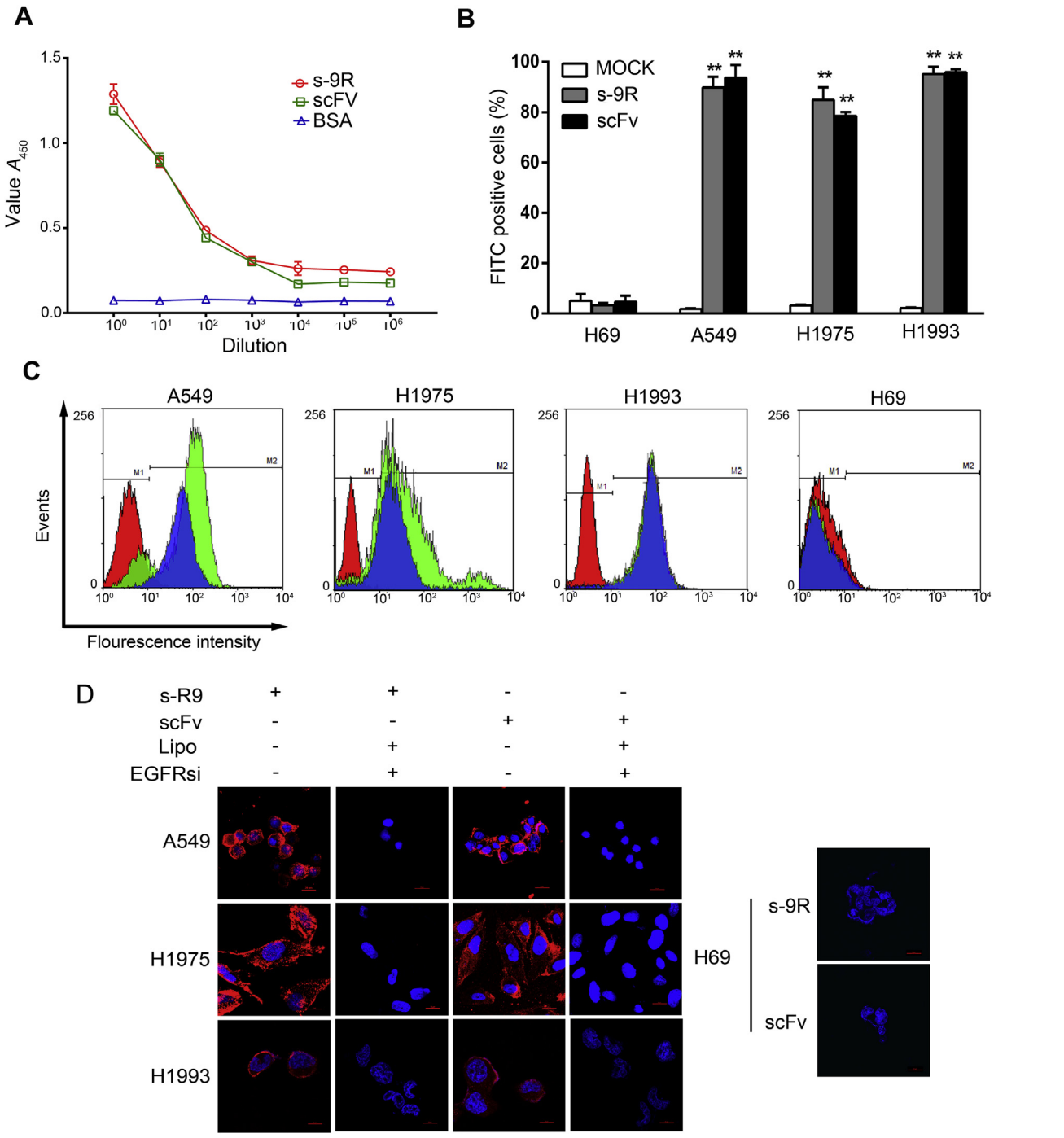

Fig. 2. Binding and internalization of the fusion proteins. (A) Detection of the EGFR-binding ability of s-9R and scFv by ELISA. (B and C) The binding of the fusion proteins to EGFR-positive NSCLC cells was assessed by an FCM assay. Lung cancer cells were incubated with PBS (red), scFv (blue) or s-9R (green) at 37 °C for 6 h. (D) Recognition of EGFR and internalization of the fusion proteins visualized by LSCM (EGFRsi: EGFR-siRNA). Lung cancer cells were incubated with the fusion proteins or BSA for 6 h before immunofluorescence staining (Red: Cy3; Blue: DAPI). Scale bar = 20 μm. A result representative of 3 independent experiments is shown, $^{**}p < 0.01$.

mutations that typically confer resistance to EGFR-TKI treatment: A549 cells carrying wild-type EGFR and the KRAS mutation [19], H1975 cells expressing L858R/T790M EGFR [20], and H1993 cells harboring a MET amplification [21]. A549, H1975 and H1993 cells were treated with complexes of scFv, s-9R or BSA and the indicated siRNA (100 nM). We evaluated the KRAS, EGFR and MET gene expression using RT-qPCR and Western blot analysis. The results showed that the mRNA expression levels of KRAS, EGFR and MET were substantially lower in the s-9R/siRNA treated groups than in the BSA/siRNA or scFv/siRNA group (Fig. 4A). Down-regulated gene expression was also confirmed with a Western blot of cells incubated for 3 days with the above treatments. As shown in Fig. 4B, similarly to the observations at the mRNA level, the siRNA delivered by s-9R efficiently inhibited the KRAS, EGFR and MET protein expression.

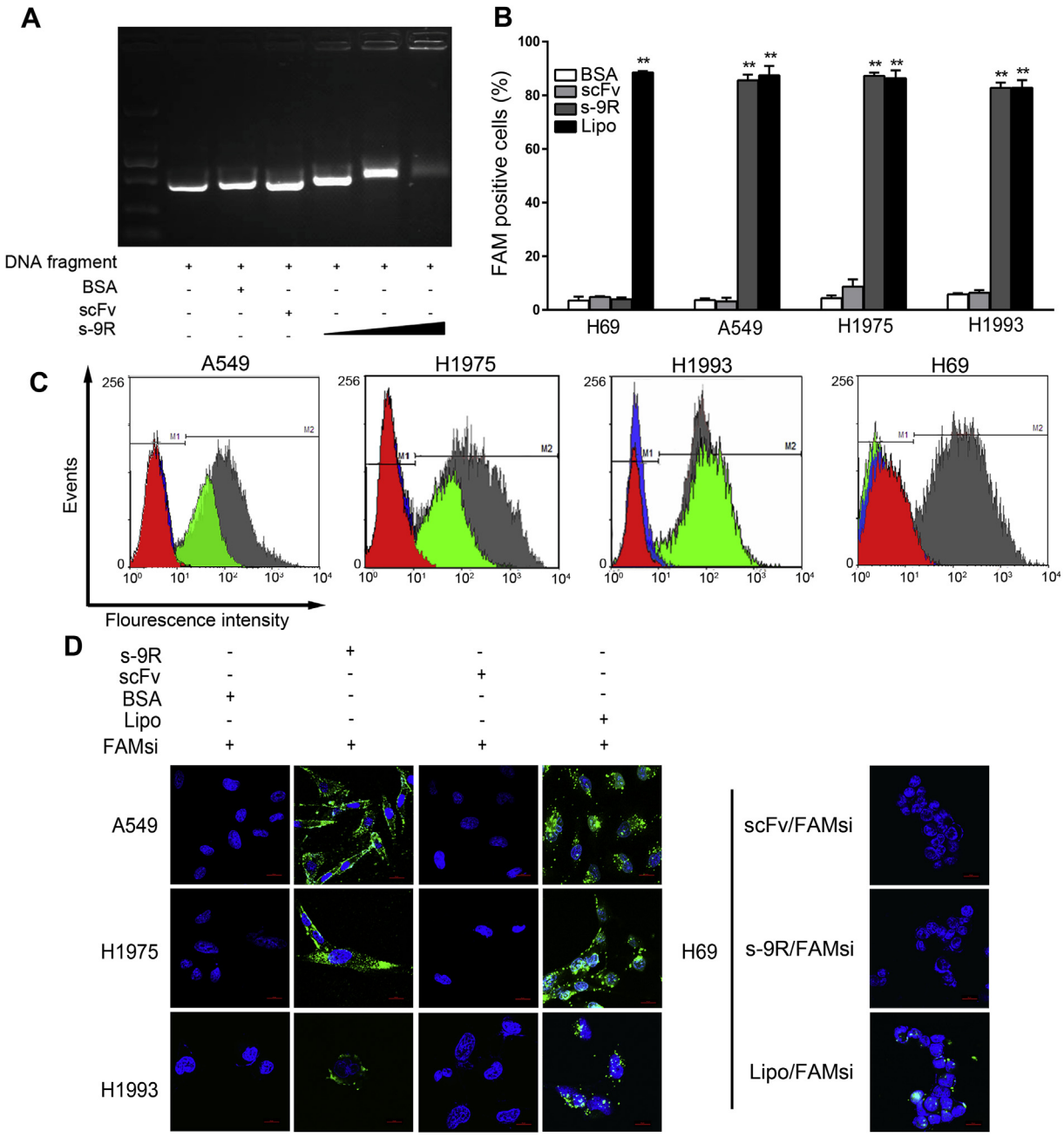

Fig. 3. siRNA can be specifically delivered into EGFR-positive NSCLC cells by the s-9R fusion protein. (A) Nucleotide binding ability of the s-9R and scFv fusion proteins determined by a gel mobility-shift assay. (B and C) FAM−siRNA (FAMsi) was pre-mixed with scFv (blue), s-9R (green), BSA (red) or Lipofectamine^{2000} (black) before being added to the medium of lung cancer cells. FAM fluorescence was detected by FCM analysis 6 h later. (D) Specific delivery of FAM-siRNA (green) visualized by LSCM. Lung cancer cells were incubated with s-9R/FAM-siRNA, scFv/FAM-siRNA or BSA/FAM-siRNA for 6 h. Cells transfected with FAM-siRNA by Lipofectamine^{2000} served as the positive control. To knockdown EGFR expression, we transfected cells with EGFR-siRNA using Lipofectamine^{2000} (Lipo) 48 h before incubation. Cells were counterstained using DAPI (blue) dye. Scale bars = 20 mm. A result representative of 3 independent experiments is shown, $^{**}p < 0.01$.

3.5. s-9R-delivered siRNA can efficiently inhibit the growth of EGFR-positive TKI-resistant NSCLC cells in vitro

The A549, H1975 and H1993 cells were treated with complexes of the fusion proteins or BSA and the indicated siRNA; gefitinib (100 nM) was also added to the cell culture medium 72 h before the cell proliferation was measured using an MTT assay. Compared with BSA/siRNA or scFv/siRNA, the siRNA delivered by s-9R significantly inhibited the EGFR-positive NSCLC cell proliferation and restored the sensitivity of the three lung cancer cells to gefitinib. Compared with treatment with either agent alone, the combination of s-9R/siRNA and gefitinib synergistically potentiated apoptosis (Fig. 4C). The proliferation ratios of the A549, H1975 and H1993 cell lines that were treated with s-9R/siRNA plus gefitinib were in the same range: 70.74 ± 7.67%, 71.72 ± 1.23% and 68.61 ± 5.64%, respectively. However, the proliferation of the cells treated with BSA/siRNA or scFv/siRNA plus gefitinib was not markedly inhibited, confirming that the reduction in cell growth was mediated by the combination of the EGFR-TKI with a specific siRNA that can be transferred to cells via either s-9R or Lipofectamine^{2000}. Cells undergoing apoptosis were also analyzed using the FCM assay. As shown in Fig. 4D, the apoptosis rate was significantly increased in the three EGFR-

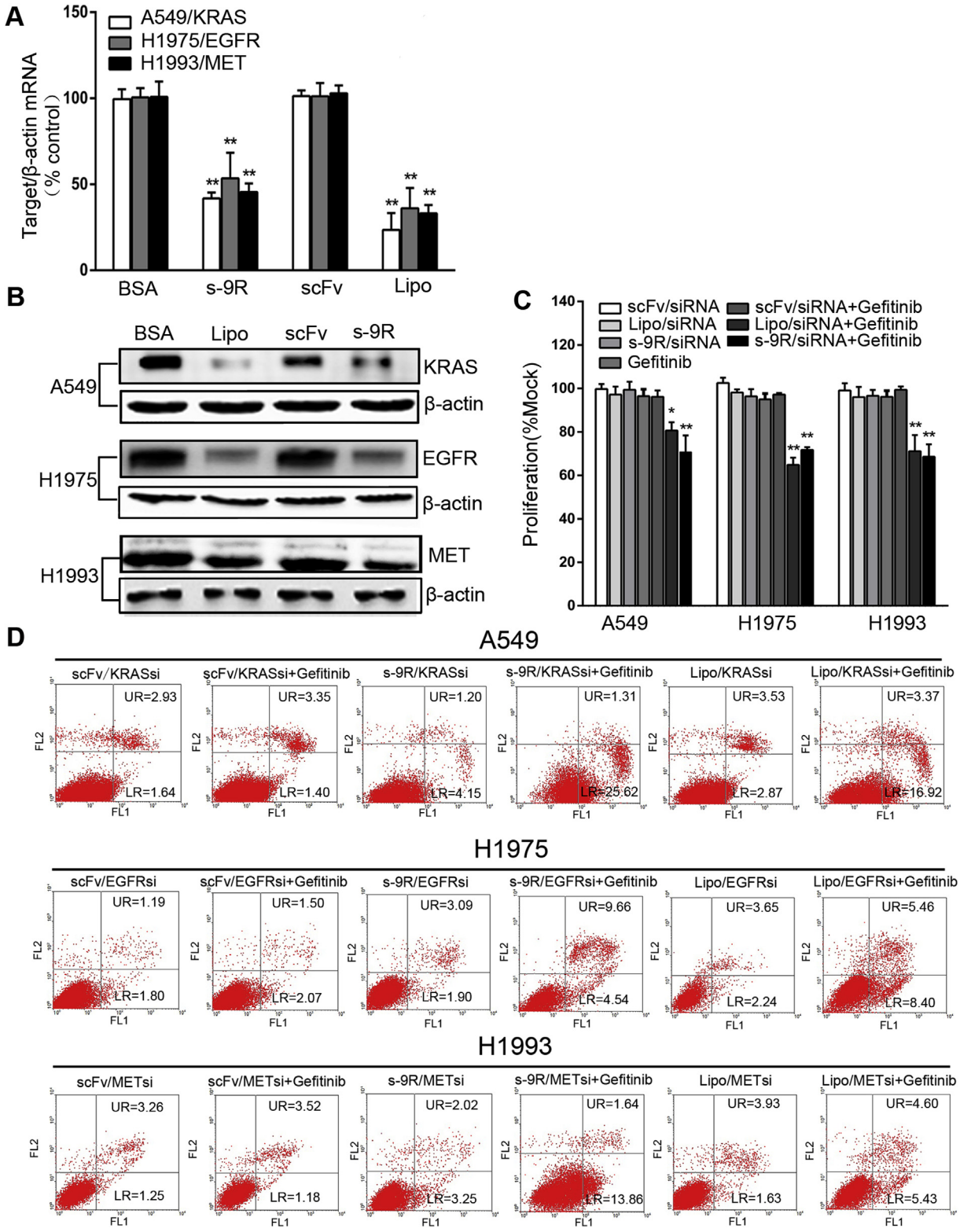

Fig. 4. siRNA delivered by s-9R reduced the targeted gene expression in EGFR-positive cells and inhibited cell proliferation. (A–B) RT-qPCR for KRAS, EGFR and MET mRNA and immunoblot analysis of KRAS, EGFR and MET proteins in A549, H1975 and H1993 cells. (C) MTT assays of A549, H1975 and H1993 cells treated as indicated. $^{*}p < 0.05$, $^{**}p < 0.01$. (D) Apoptosis analysis of EGFR-positive cells treated as indicated assessed by FCM.

positive cells treated with the s-9R/siRNA plus gefitinib. However, the apoptosis rate of cells treated with scFv/siRNA alone or in combination with gefitinib did not markedly change. Therefore, s-9R/siRNA restored the sensitivity of EGFR-positive NSCLC cells to gefitinib by silencing the expression of resistance-related genes.

3.6. s-9R-delivered, EGFR-specific siRNA suppresses tumor growth in vivo

To visualize the EGFR tumor targeting, the fusion proteins were conjugated to DyLight 800 dye for nude mouse studies. The DyLight

800 conjugates showed a distinct absorbance peak at 780 nm (Fig. S2). The distribution of the intravenously injected, DyLight 800-labeled scFv and s-9R was examined in lung cancer cells implanted nude mice using a whole-body near-infrared fluorescence living imaging (FLI) system. After the xenografts grew locally around the injection site, we injected DyLight 800-labeled scFv, s-9R or BSA via the tail vein. As demonstrated in Fig. 5A and B, three dye-labeled proteins diffused rapidly throughout the whole bodies of H1975 tumor-bearing mice via the venous system in 1 h. Eight hours later, the fusion proteins were gradually cleared from the normal tissues and were enriched in the tumor tissues until 48 h. In contrast, BSA was cleared from the body 8 h post-injection, and fluorescence was detected mainly in the kidneys but not in the tumor tissues. During the whole observation period, fluorescence of DyLight 800-conjugated proteins did not accumulate in the H69 tumor tissues. The distribution of the intravenously-injected protein/siRNA complexes was also evaluated in model nude mice using FLI. We injected Cy5-siRNA (2′-O-me modified) pre-mixed with BSA, scFv or s-9R via the tail vein. We observed that the Cy5-siRNA mixed with s-9R was intracorporeally distributed in the same pattern as the single-chain antibody alone. As shown in Fig. 6A, the venous system distributed fluorescence throughout the entire body in every group 1 h after injection in the H1975-tumor-bearing mice. Eight hours later, the s-9R/Cy5-siRNA complexes were gradually cleared from the normal tissues and were enriched in the tumor tissues until 48 h. In contrast, the siRNA mixed with BSA or scFv was mainly localized in the kidney until 48 h after the injection, presumably because these components were eliminated by the kidney. Similarly, Cy5 fluorescence did not accumulate in any of the H69 cell tumors during the entire observation period, and it rapidly dissipated. A significant fluorescent signal was not detected in the H1975-tumor-bearing mice receiving BSA/Cy5-siRNA or scFv/Cy5-siRNA. Forty-eight hours after the injection, we harvested tumors and organs to assess the distribution of the exclusively EGFR-positive tumor. As demonstrated in Fig. 6B and Fig. S3, the fluorescence was concentrated in the H1975 tumors treated with s-9R/Cy5-siRNA but was not detected in the H69 tumors, indicating that s-9R targets EGFR. Weak signals were detected in the liver and spleen, and the fluorescence accumulation in the heart, muscle and lung was negligible. Notably, a relatively strong fluorescence signal showed accumulation in the kidney of the H1975-tumor-bearing mice receiving s-9R/Cy5-siRNA 48 h after injection. To confirm the intracorporeal distribution of s-9R/siRNA, we injected BSA, scFv or s-9R/FAM-siRNA (2′-O-me modified) complexes into the tail vein. Forty-eight hours later, the frozen xenografts sections were prepared and examined under an LSCM. As shown in Fig. 6C, green fluorescence was detected in the H1975 tumor sections treated with the s-9R/FAM-siRNA complex, but not in the H1975 tumor sections receiving BSA/FAM-siRNA or scFv/FAM-siRNA or in any of the H69 tumor sections.

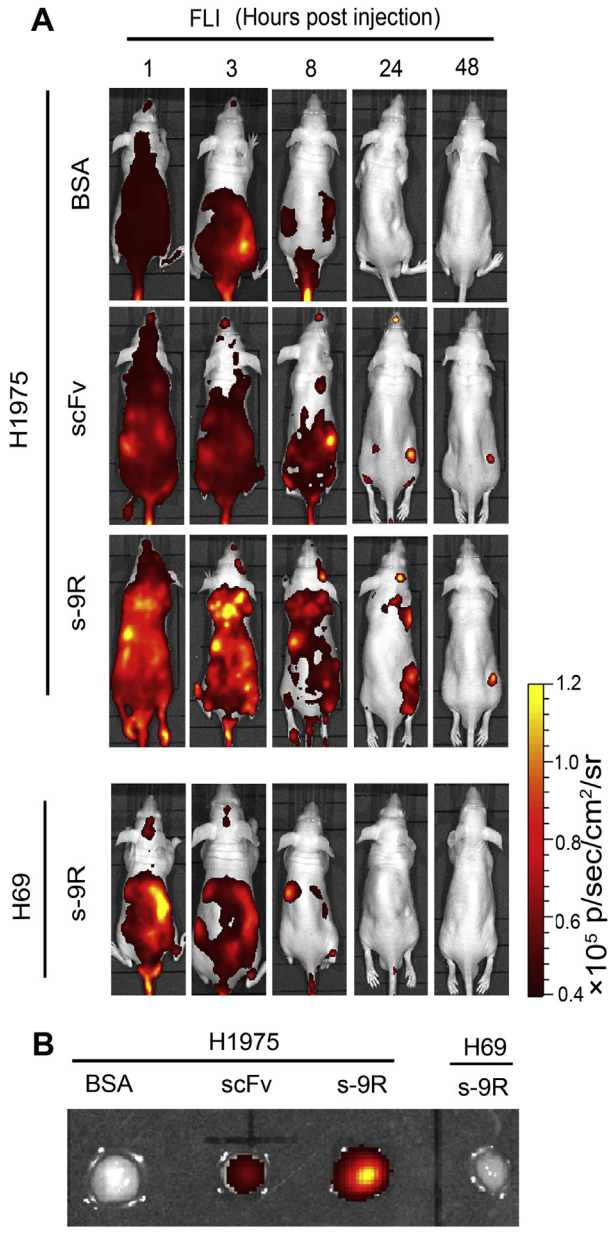

Fig. 5. *In vivo* distribution of scFv and s-9R in H1975 and H69 tumor-bearing mice. (A) Whole-body images of the fusion protein distribution. Images obtained at indicated time intervals after injection. (B) Fluorescence images of tumor sections.

We next investigated the *in vivo* antitumor activity of the s-9R/siRNA complex. The mice bearing H1975 tumors received intravenous injections of s-9R or the scFv/EGFR-siRNA complex twice per week for 6 weeks, whereas a PBS solution of BSA/EGFR-siRNA was used as a negative control. In addition, gefitinib was intragastrically administered to nude mice. As observed in the immunohistochemistry (IHC) analysis of tumor sections from the s-9R/EGFR-siRNA plus gefitinib group, the H1975 xenografts showed reduced EGFR staining. Furthermore, the cell proliferation, as assessed based on the proportion of Ki-67-positive cells, decreased from >60% in the control groups to 17.67 ± 2.52% in the treated groups. Significantly enhanced apoptosis was also observed by assessing the proportion of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)-positive cells, which increased from approximately 2% in the control groups to 68.33 ± 4.16% in the treated groups. However, the same treatment did not significantly alter these parameters in the EGFR-deficient H69 xenografts (Fig. 7A and B). Compared to the control groups that received scFv or BSA/EGFR-siRNA complexes plus gefitinib oral gavage, the H1975 tumor growth was markedly suppressed by the s-9R/EGFR-siRNA plus gefitinib treatment, and tumors were not readily detected, as indicated by their negligible weight and size (Fig. 8A and B). Two of seven mice treated with these combination therapies did not develop measurable tumors. Moreover, a Kaplan–Meier survival analysis revealed that mice bearing H1975 xenografts and treated with s-9R/EGFR-siRNA plus gefitinib survived longer than the mice in the other groups, including the H69 xenograft-bearing mice treated with the same drug combination (Fig. 8C). This finding further supports the potent effect of the treatment with a targeted siRNA plus an oral TKI. Therefore, we

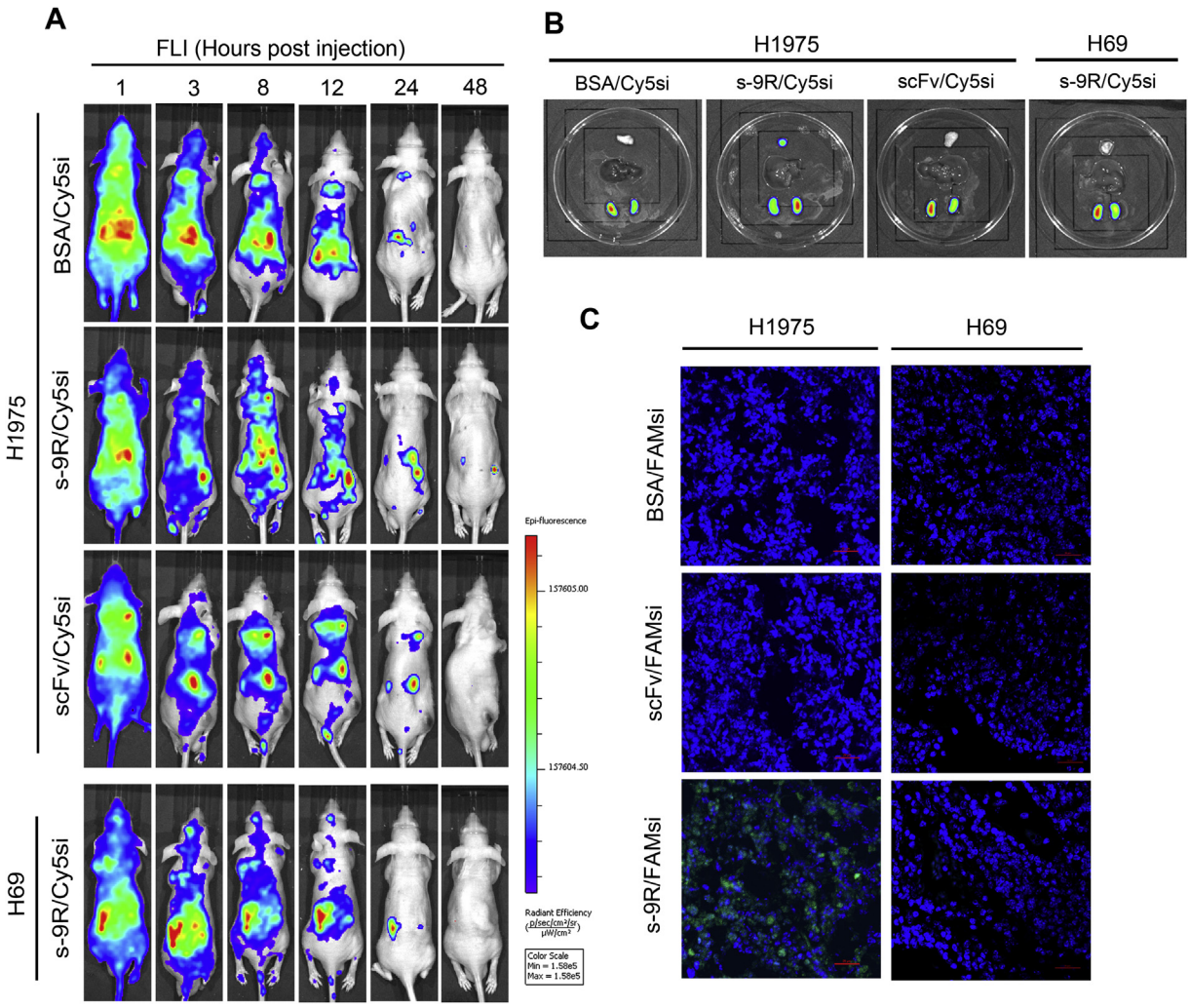

Fig. 6. *In vivo* targeted delivery of siRNA mediated by s-9R. (A) Whole-body images of the distribution of Cy5-siRNA, which was pre-mixed with the fusion proteins or BSA. Images obtained at indicated time intervals after injection. (B) Fluorescence images of tumor sections. (C) Mice treated with protein/FAM-siRNA before necropsy. Fluorescence images of tumor cryosections were taken by LSCM. Scale bar = 20 μm.

conclude that the suppression of EGFR-positive tumor growth by the s-9R/EGFR-siRNA plus gefitinib treatment was likely mediated by the inhibition of tumor cell proliferation and the induction of apoptosis due to selective EGFR gene knockdown.

3.7. *Expression and clinico-pathological correlation of EGFR in human NSCLC specimens*

To assess the expression levels of EGFR and its effect on prognosis, we screened 75 human NSCLC specimens and analyzed the correlation between EGFR and clinico-pathological parameters as well as follow-up information. As shown in Table 1, 44 cases of EGFR-positive NSCLC accounting for 58.66% of the available NSCLC cases were examined, including 37 adenocarcinomas, 32 squamous cell carcinomas and 6 large cell lung cancers. The median survival time of these patients was 16.3 months. Twenty-six patients died within 6 months (34.7%), and the specimens from these patients were all EGFR-positive; 18 patients died within 1 year of the diagnosis (24%) and 15 of these specimens were EGFR-positive (83.3%); 31 survived more than 1 year (41.3%), and only 3 samples from these patients were EGFR-positive (9.7%). More importantly, positive EGFR expression in NSCLC significantly correlated with lymph node metastasis ($p = 0.005$) and distant metastasis ($p = 0.013$), which suggests that EGFR-positive patients may benefit from an adjuvant immuno-drug strategy.

4. Discussion

Patients with NSCLC who initially respond to reversible EGFR inhibitors almost invariably develop resistance to EGFR TKIs and relapse after several months. This problem is thought to be due to the secondary mutation of EGFR or a kinase-mediated bypass mechanism associated with the activation of downstream signaling pathways of EGFR [6]. Several new agents, so-called secondary generation inhibitors, have been developed as an irreversible pan-EGF receptor TKI to overcome EGFR-TKI resistance and prolong responses. However, the toxicities associated with irreversible inhibition of wild-type EGFR were so severe as to lead to dose reductions or treatment termination [22]. Third-generation inhibitors that spared wild-type EGFR were developed and have shown promising clinical activity [23]. However, the co-occurrence of multiple resistance mechanisms, such as EGFR T790M mutation and MET amplification, within a given tumor sample or among different sites of disease limits the therapeutic efficacy of mutant-specific EGFR inhibitors [10]. Moreover, mechanisms that lead to direct resistance against these novel inhibitors in NSCLC cells have

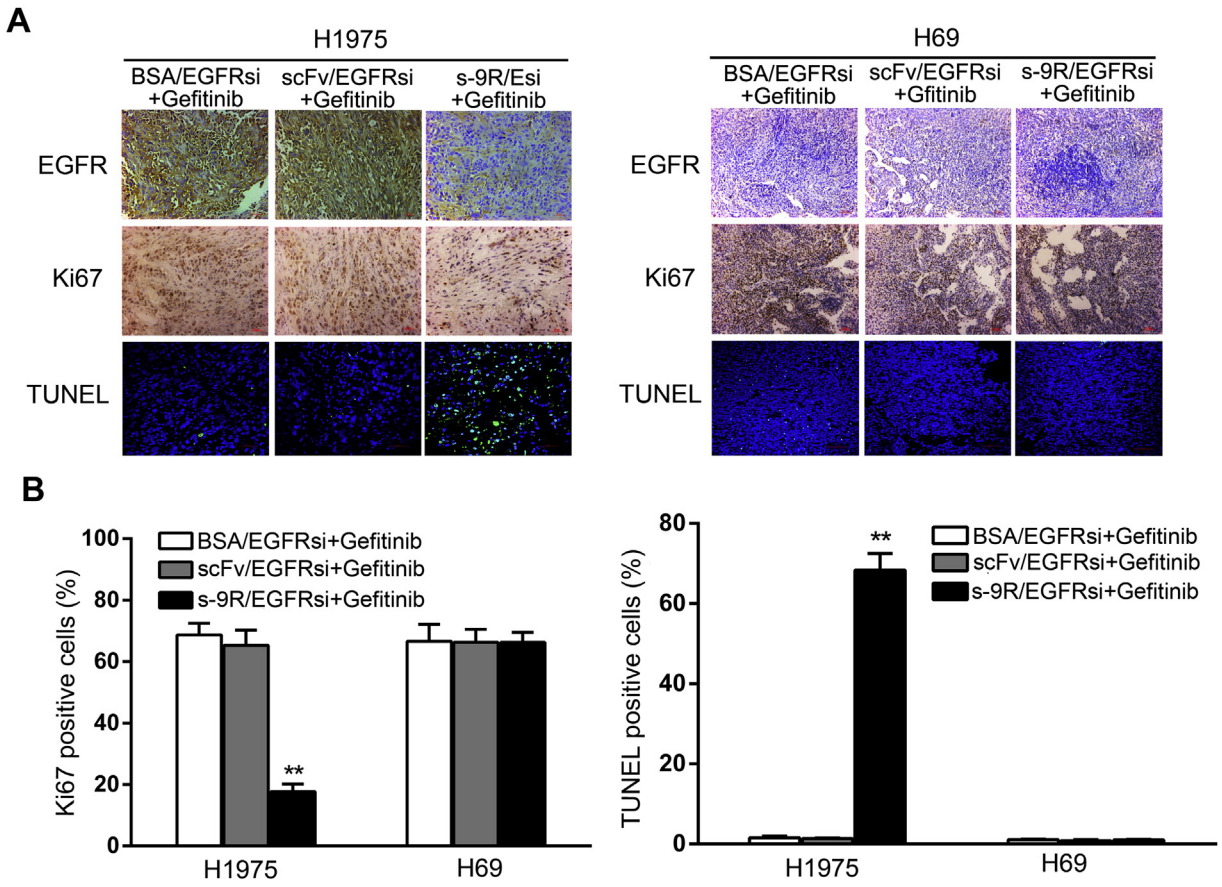

Fig. 7. Intravenous injection of s-9R/EGFR-siRNA reduced EGFR expression and induced apoptosis in EGFR-positive tumors by restoring gefitinib sensitivity. (A) Nude mice bearing H1975 or H69 xenografts were treated with protein/siRNA and gefitinib. Representative microscopy images (×200) of tumor sections stained for EGFR, Ki67 and TUNEL are shown. Scale bar = 50 μm. (B) Percentages of TUNEL- or Ki67-positive cells from six randomly selected fields of paraffin-embedded tumor sections. $^{**}p < 0.01$.

been identified in preclinical studies [9,24]. Other strategies for overcoming reversible EGFR-TKI resistance involve the simultaneous blocking of parallel signaling pathways. Mutant KRAS protein is constitutively activated, which leads to stimulus-independent, persistent activation of downstream effectors and consequently resistance to EGFR inhibitors [25]. However, not all cancer cells that harbor RAS mutations depend on mutant RAS to survive; inhibiting KRAS by itself might be insufficient to terminate cancer growth and progression [26]. More importantly, inhibitors targeting KRAS signaling block KRAS and NRAS geranylgeranylation, have severe toxicity [27]. MET can cross-talk with ERBB3, a member of the EGFR family, and consequently stimulate the ERBB3-dependent activation of PI3K/AKT signaling in an EGFR-independent manner [28]. Inhibitors of the MET protein have been tested in clinical trials, but no compelling evidence of a clinical benefit was obtained in NSCLC patients whose initial treatment with TKIs failed [29].

Small RNAs, including EGFR-specific siRNAs and multi-target microRNAs, have been shown to strongly synergize with EGFR-TKIs to inhibit the proliferation of lung cancer cells [30,31]. The success of siRNA therapy depends on the optimization of site-specific transportation, serum stability and cellular uptake, which has hindered its application in the clinic. Recently, Jiang, et al. reported an anti-HER2 single-chain antibody (e23sFv-9R) that can deliver CXCR4-siRNA to breast cancer cells to exert anti-tumor effects [32]. In the present study, we designed and produced two new EGFR-specific scFvs in *E. coli* using the amino acid sequence of nimotuzumab, which is a well-established anti-EGFR mAb possessing potent antitumor immuno-activity, and is being investigated in several clinical trials for the treatment of malignancies [33–35]. A previous study used nimotuzumab conjugated to isotopes to detect EGFR-positive breast cancer, and showed that its conjugates can bind to EGFR and can be internalized and transported to the cytoplasm and nuclei of EGFR-positive cancer cells [36]. Considering the specific binding and internalization of nimotuzumab, we speculated that nimotuzumab derivatives might deliver siRNA into the cytoplasm of target cells and specifically silence target genes. In addition, considering the molecular weight and influence on the protein spatial structure, we selected 9R rather than protamine as the siRNA carrier. Data showed that 9R did not affect the selectivity of the scFv for EGFR-positive NSCLC cells. Additionally, to increase the serum stability and reduce off-target activity, we modified the seed region of the siRNA via 2′-O-methylation, which did not compromise the silencing ability of siRNA [12]. Generally, our data showed that the new s-9R fusion protein can selectively deliver siRNA into EGFR-TKI-resistant NSCLC cells and efficiently silence target gene expression. When co-administered with gefitinib, the siRNA delivered by s-9R effectively inhibited the proliferation of resistant NSCLC cells, induced apoptosis and enhanced the xenograft tumor response to gefitinib. The EGFR-TKI treatment amplifies the suppression of tumor cell growth and the induction of apoptosis by siRNA. *In vitro*, neither the EGFR-siRNA that was transfected using Lipofectamine^{2000} nor that delivered using s-9R as a monotherapy efficiently suppressed the viability of H1975 cells; these findings are slightly inconsistent with previous data [30]. These differences might be the result of the lower concentration of siRNA used in our study and the ability of

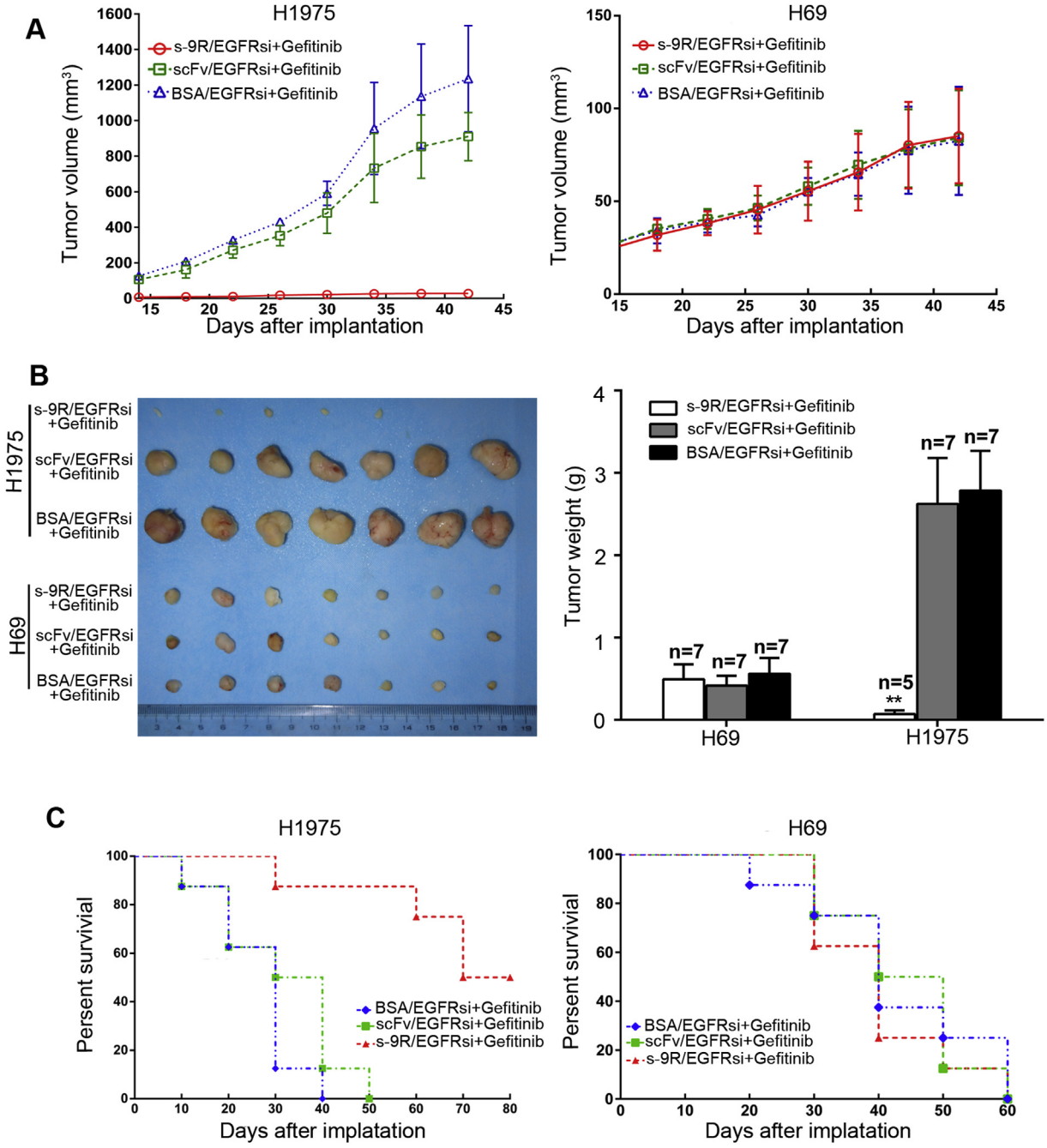

Fig. 8. Inhibited tumor growth and prolonged life span of mice bearing H1975 xenografts. (A) Tumor volumes are presented as growth curves, and (B) digital pictures of the tumors were recorded in each group; the tumors were weighed after sacrifice. Data are expressed as the means ± S.D. for each group. $^{**}p < 0.01$. (C) Kaplan–Meier survival curve.

the siRNAs to silence gene expression. Furthermore, the EGFR in H1975 cells, which carries a T790M mutation plus an L858R mutation in exon 21, is considered a less potent driver of cell growth and survival than the EGFR in TKI-resistant cell lines that carry an exon 19 deletion. Interestingly, the rates of apoptosis induced by the s-9R-mediated siRNA delivery or Lipofectamine^{2000}-mediated siRNA transfection did not differ in the presence of gefitinib; furthermore, the latter strategy more efficiently silenced gene expression than did the former. We suppose that the antigen-blocking capacity of scFv may play an important part in promoting apoptosis. Cancer cells are targeted by three mechanisms: the scFv moiety prevents the receptor dimerization, the receptor was then physically eliminated by EGFR-targeted siRNA, and gefitinib inhibits the enzymatic activity of EGFR signaling pathways.

KRAS and EGFR in human lung tumors activate common signaling pathways to support tumorigenesis. A previous report showed that KRAS knockdown by a specific siRNA restored TKI sensitivity in KRAS-activated A549 cells [37]. Mutant KRAS knockdown increased the sensitivity of NSCLCs with wild-type EGFR to gefitinib by necessitating the need for signaling via the ligand-dependent activation and phosphorylation of EGFR and thus increasing survival [38]. In our study, KRAS ablation did not significantly diminish the viability of A549 cells, which is consistent with the findings of other studies, whereas the combination

Table 1
Correlation between clinico-pathological parameters and EGFR expression.

Feature	N	EGFR positive (%)	P value
Age (Year)			0.483
<60	36	27(75)	
≥60	39	17(43.6)	
Sex			0.979
Male	51	29(56.9)	
Female	24	15(62.5)	
Pathologic type			0.089
Adenocarcinma	37	25 (67.6)	
Squamous cell carcinoma	32	17(53.1)	
Others	6	2(33.3)	
Lymph node metastasis			0.005
No	16	4(25)	
Yes	59	40(67.8)	
Distant metastasis			0.013
No	30	9(30)	
Yes	45	35(77.8)	
Survival time (months)			0.000
<6	26	26(100)	
>6, <12	18	15(83.3)	
≥12	31	3(9.7)	

strategy successfully restored the sensitivity of A549 cells to EGFR TKIs [39]. However, constitutive MET phosphorylation is insufficient to induce EGFR-TKI resistance in NSCLC cells, and further activation by HGF or MET amplification might be necessary [40]. A previous study showed that MET downregulation can sensitize EGFR-TKI-resistant lung cancer cells to gefitinib [41]. In the present study, we used s-9R to deliver MET-specific siRNA into TKI-resistant H1993 cells to specifically knockdown MET expression. By silencing MET gene expression, we reduced the amount of MET on the cell surface to a level far below the threshold level for TKI resistance by preventing the activation of downstream PI3K/Akt-pathway effectors.

Drug combination strategies, including rational combinations of target therapy, chemotherapy and immunotherapy that block various targets, can augment the potency of EGFR signaling inhibition and suppress TKI-resistant cancer cells. Nevertheless, improving long-term survival outcomes without amplified toxicities is a major goal for combination therapies that are designed to circumvent resistance. Taking advantage of the ability of s-9R to deliver siRNAs directly to cancer cells *in vivo*, we demonstrated that the combination of immunotherapy and small molecular inhibitors more effectively inhibited the tumor in the model system than did the control treatments. Our results provide preclinical support for the therapeutic potential of s-9R/siRNA, which has great potential to restore the sensitivity of EGFR-TKI-resistant NSCLC cells and to avoid non-specific side-effects.

scFv/siRNA complexes hold great promise as therapeutic agents for NSCLC through the application of RNAi to oncogene expression. However, some problems still must be solved before our strategy can be applied clinically. First, because the siRNA sequences in this study target both the mutated and wild-type EGFR and KRAS mRNA, future work is needed to investigate the possibility of a specific knockdown of the mutated EGFR and KRAS by designing mutant-specific siRNAs. Second, the antibody must be modified further to optimize the loading capacity of the nucleic acids and the binding stability between scFv and siRNA by fusing them with other positively charged peptides or adopting artificial polymers. Third, the release of siRNA from endosomes should be accelerated by fusion with motifs that exert endosomolytic activity. Finally, although the antibody fusion protein complexed with siRNA does not induce interferon or activate other nonspecific inflammatory responses when administered *in vivo* [12,32,42], we did not observe any obvious toxicity or inflammatory infiltrate in our tumor-bearing mouse model. This result needs to be investigated more carefully and verified in a future study.

Our strategy may benefit NSCLC patients harboring wild-type EGFR, who account for approximately 60% of East Asians and 85% of Caucasians, who are now excluded from EGFR-TKI treatment [28]. Patients harboring more than one resistant mutation in a single tumor site may also benefit from our method because the s-9R could be used to deliver any mixture of siRNAs that target different genes associated with EGFR-TKI resistance. Furthermore, EGFR is overexpressed in a variety of epithelial tumors [43]. Small molecular inhibitors, mostly RTK inhibitors, have been widely used in clinics to treat these tumors, but their effectiveness has been hampered by the emergence of drug resistance. Theoretically, our strategy can be easily adapted to sensitize these EGFR-positive tumor cells by sensibly silencing target genes associated with drug resistance. Last but not least, the antibody-mediated EGFR-specific delivery systems might be useful for the future development of multifunctional vesicles for simultaneous target imaging, drug delivery and real-time monitoring of the therapeutic effect to treat EGFR-overexpressed cancers and other diseases by conjugation with magnetic nanoparticles.

5. Conclusions

The strategy of using an scFv antibody to deliver siRNA might be useful for overcoming TKI resistance in EGFR-overexpressing NSCLC patients whose therapy fails due to intrinsic or acquired resistance. In the present work, we generated two EGFR-specific scFvs and confirmed their binding and internalization capacity. We efficiently silenced the expression of targeted genes by utilizing s-9R, which is internalized by NSCLC cell lines, as an siRNA carrier. Co-treatment with s-9R/siRNAs and gefitinib successfully induced apoptosis in TKI-resistant NSCLC cells *in vitro* and suppressed xenograft tumor progression *in vivo*. The synthesis and application of new small-molecule inhibitors will likely result in novel causes of TKI resistance. Thus, we could restore the TKI-resistant cancer cell sensitivity by selecting suitable targets for knockdown. This scFv antibody-mediated siRNA delivery system and the combination strategy can increase the possibility of using siRNA therapeutically to overcome EGFR-TKI resistance in clinical applications.

Acknowledgments

The National Natural Science Foundation of China (No.81172222) and the Social Development Project Foundation of Shaanxi Province (No.2011K15-06-02) are gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.10.036.

References

[1] N.E. Hynes, H.A. Lane, ERBB receptors and cancer: the complexity of targeted inhibitors, Nat. Rev. Cancer 5 (2005) 341–354.
[2] G. Giaccone, Y. Wang, Strategies for overcoming resistance to EGFR family tyrosine kinase inhibitors, Cancer Treat. Rev. 37 (2011) 456–464.
[3] C.H. Yun, K.E. Mengwasser, A.V. Toms, M.S. Woo, H. Greulich, K.K. Wong, et al., The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 2070–2075.
[4] William Pao, Theresa Y. Wang, Gregory J. Riely, Vincent A. Miller, Qiulu Pan, Marc Ladanyi, et al., KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib, PLoS Med. 2 (2005) e17.
[5] J.A. Engelman, K. Zejnullahu, T. Mitsudomi, Y. Song, C. Hyland, J.O. Park, et al., MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling, Science 316 (2007) 1039–1043.

[6] C. Rolfo, E. Giovannetti, D.S. Hong, T. Bivona, L.E. Raez, G. Bronte, et al., Novel therapeutic strategies for patients with NSCLC that do not respond to treatment with EGFR inhibitors, Cancer Treat. Rev. 40 (2014) 990–1004.
[7] Lorenza Landi, F. Cappuzzo, Irreversible EGFR-TKIs: dreaming perfection, Transl. Lung Cancer Res. 2 (2013) 40–49.
[8] D. Ercan, K. Zejnullahu, K. Yonesaka, Y. Xiao, M. Capelletti, A. Rogers, et al., Amplification of EGFR T790M causes resistance to an irreversible EGFR inhibitor, Oncogene 29 (2010) 2346–2356.
[9] K.S. Thress, C.P. Paweletz, E. Felip, B.C. Cho, D. Stetson, B. Dougherty, et al., Acquired EGFR C797S mutation mediates resistance to AZD9291 in non-small cell lung cancer harboring EGFR T790M, Nat. Med. 21 (2015) 560–562.
[10] H.A. Yu, G.J. Riely, C.M. Lovly, Therapeutic strategies utilized in the setting of acquired resistance to EGFR tyrosine kinase inhibitors, Clin. Cancer Res. 20 (2014) 5898–5907.
[11] S.Y. Wu, G. Lopez-Berestein, G.A. Calin, A.K. Sood, RNAi therapies: drugging the undruggable, Sci. Transl. Med. 6 (2014) 240ps7.
[12] Y.D. Yao, T.M. Sun, S.Y. Huang, S. Dou, L. Lin, J.N. Chen, et al., Targeted delivery of PLK1-siRNA by ScFv suppresses Her2+ breast cancer growth and metastasis, Sci. Transl. Med. 4 (2012) 130ra48.
[13] W.-H. Wen, J.-Y. Liu, W.-J. Qin, J. Zhao, T. Wang, L.-T. Jia, et al., Targeted inhibition of HBV gene expression by single-chain antibody mediated small interfering RNA delivery, Hepatology 46 (2007) 84–94.
[14] F. Wang, H.R. Bai, J. Wang, Y.Z. Bai, C.W. Dou, Glioma growth inhibition in vitro and in vivo by single chain variable fragments of the transferrin receptor conjugated to survivin small interfering RNA, J. Int. Med. Res. 39 (2011) 1701–1712.
[15] P. Kumar, H.S. Ban, S.S. Kim, H. Wu, T. Pearson, D.L. Greiner, et al., T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice, Cell. 134 (2008) 577–586.
[16] Maria Valentina Pasquetto, Luca Vecchia, Daniele Covini, Rita Digilio, C. Scotti, Targeted drug delivery using immunoconjugates: principles and applications, J. Immunother. 34 (2011) 611–628.
[17] P.A. Wender, D.J. Mitchell, K. Pattabiraman, E.T. Pelkey, L. Steinman, J.B. Rothbard, The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 13003–13008.
[18] R. Siegel, J. Ma, Z. Zou, A. Jemal, Cancer statistics, 2014, CA Cancer J. Clin. 64 (2014) 9–29.
[19] T. Li, Y.H. Ling, I.D. Goldman, R. Perez-Soler, Schedule-dependent cytotoxic synergism of pemetrexed and erlotinib in human non-small cell lung cancer cells, Clin. Cancer Res. 13 (2007) 3413–3422.
[20] R. Sordella, D.W. Bell, D.A. Haber, J. Settleman, Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways, Science 305 (2004) 1163–1167.
[21] P.C. Ma, R. Jagadeeswaran, S. Jagadeesh, M.S. Tretiakova, V. Nallasura, E.A. Fox, et al., Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer, Cancer Res. 65 (2005) 1479–1488.
[22] L.V. Sequist, J.C. Yang, N. Yamamoto, K. O'Byrne, V. Hirsh, T. Mok, et al., Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations, J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 31 (2013) 3327–3334.
[23] P.A. Janne, J.C. Yang, D.W. Kim, D. Planchard, Y. Ohe, S.S. Ramalingam, et al., AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer, N. Engl. J. Med. 372 (2015) 1689–1699.
[24] C.A. Eberlein, D. Stetson, A.A. Markovets, K.J. Al-Kadhimi, Z. Lai, P.R. Fisher, et al., Acquired resistance to mutant-selective EGFR inhibitor AZD9291 is associated with increased dependence on RAS signaling in preclinical models, Cancer Res. 75 (2015) 2489–2500.
[25] P.J. Roberts, T.E. Stinchcombe, KRAS mutation: should we test for it, and does it matter? J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. 31 (2013) 1112–1121.
[26] K. Suda, K. Tomizawa, T. Mitsudomi, Biological and clinical significance of KRAS mutations in lung cancer: an oncogenic driver that contrasts with EGFR mutation, Cancer Metastasis Rev. 29 (2010) 49–60.
[27] J. Downward, Targeting RAS signalling pathways in cancer therapy, Nat. Rev. Cancer 3 (2003) 11–22.
[28] J.A. Engelman, P.A. Janne, Mechanisms of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer, Clin. Cancer Res. 14 (2008) 2895–2899.
[29] G. Scagliotti, J. von Pawel, S. Novello, R. Ramlau, A. Favaretto, F. Barlesi, et al., Phase III multinational, randomized, double-blind, placebo-controlled study of tivantinib (ARQ 197) plus erlotinib versus erlotinib alone in previously treated patients with locally advanced or metastatic nonsquamous non–small-cell lung cancer, J. Clin. Oncol. 33 (2015) 2667–2674.
[30] G. Chen, P. Kronenberger, E. Teugels, I.A. Umelo, J. De Greve, Targeting the epidermal growth factor receptor in non-small cell lung cancer cells: the effect of combining RNA interference with tyrosine kinase inhibitors or cetuximab, BMC Med. 10 (2012) 28.
[31] C. Stahlhut, F.J. Slack, Combinatorial Action of MicroRNAs let-7 and miR-34 effectively synergizes with erlotinib to suppress non-small cell lung cancer cell proliferation, Cell Cycle 14 (2015) 2171–2180.
[32] K. Jiang, J. Li, J. Yin, Q. Ma, B. Yan, X. Zhang, et al., Targeted delivery of CXCR4-siRNA by scFv for HER2 breast cancer therapy, Biomaterials 59 (2015) 77–87.
[33] U. Bode, M. Massimino, F. Bach, M. Zimmermann, E. Khuhlaeva, M. Westphal, et al., Nimotuzumab treatment of malignant gliomas, Expert Opin. Biol. Ther. 12 (2012) 1649–1659.
[34] M. Sundvall, A. Karrila, J. Nordberg, R. Grenman, K. Elenius, EGFR targeting drugs in the treatment of head and neck squamous cell carcinoma, Expert Opin. Emerg. Drugs 15 (2010) 185–201.
[35] C. Gridelli, P. Maione, M.L. Ferrara, A. Rossi, Cetuximab and other anti-epidermal growth factor receptor monoclonal antibodies in the treatment of non-small cell lung cancer, Oncologist 14 (2009) 601–611.
[36] A. Fasih, H. Fonge, Z. Cai, J.V. Leyton, I. Tikhomirov, S.J. Done, et al., ^{111}In-Bn-DTPA-nimotuzumab with/without modification with nuclear translocation sequence (NLS) peptides: an Auger electron-emitting radioimmunotherapeutic agent for EGFR-positive and trastuzumab (Herceptin)-resistant breast cancer, Breast Cancer Res. Treat. 135 (2012) 189–200.
[37] P.P. Luk, P. Galettis, M. Links, ERK phosphorylation predicts synergism between gemcitabine and the epidermal growth factor receptor inhibitor AG1478, Lung Cancer 73 (2011) 274–282.
[38] N. Sunaga, D.S. Shames, L. Girard, M. Peyton, J.E. Larsen, H. Imai, et al., Knockdown of oncogenic KRAS in non-small cell lung cancers suppresses tumor growth and sensitizes tumor cells to targeted therapy, Mol. Cancer Ther. 10 (2011) 336–346.
[39] A. Singh, P. Greninger, D. Rhodes, L. Koopman, S. Violette, N. Bardeesy, et al., A gene expression signature associated with "K-Ras addiction" reveals regulators of EMT and tumor cell survival, Cancer Cell 15 (2009) 489–500.
[40] B. Lutterbach, Q. Zeng, L.J. Davis, H. Hatch, G. Hang, N.E. Kohl, et al., Lung cancer cell lines harboring MET gene amplification are dependent on Met for growth and survival, Cancer Res. 67 (2007) 2081–2088.
[41] W. Wang, Q. Li, S. Takeuchi, T. Yamada, H. Koizumi, T. Nakamura, et al., Met kinase inhibitor E7050 reverses three different mechanisms of hepatocyte growth factor-induced tyrosine kinase inhibitor resistance in EGFR mutant lung cancer, Clin. Cancer Res. 18 (2012) 1663–1671.
[42] E. Song, P. Zhu, S.K. Lee, D. Chowdhury, S. Kussman, D.M. Dykxhoorn, et al., Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors, Nat. Biotechnol. 23 (2005) 709–717.
[43] C.L. Arteaga, Overview of epidermal growth factor receptor biology and its role as a therapeutic target in human neoplasia, Semin. Oncol. 29 (2002) 3–9.